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EXAMINATION OF 8-Cl-cAMP GENOTOXICITY BY TWO IN VIVO TEST IN BALB/c STRAIN MICE

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The antitumor agent 8-Cl-cAMP (8-chloro-cyclic adenosine monophosphate) is the most potent site-selective analogue of cAMP. It acts primarily by selective down-regulation of regulatory sub-units of cAMP-dependent protein kinases. This results in reversion of the neoplastic predominance of PK-I type over PK-II type protein kinase back to the ratio more typical to of the normal phenotype. The differential activity of 8-Cl-cAMP towards protein kinase isozymes leads to inhibition of cell growth, differentiation and neoplastic reversion of a wide variety of cancer cell lines. Since 8-Cl-cAMP has been investigated as a new potential anticancer drug with no previous studies of its mutagenic and clastogenic effects, we have investigated the genotoxicity of 8-Cl-cAMP.

Genotoxic effects were estimated by the bone marrow micronucleus assay and the occurrence of morphological chromosome lesions in adult mice (BALB/c strain). 8-Cl-cAMP was administered intraperitoneally (i.p) in three doses, 10 mg/kg b.w.; 90 mg/kg b.w. and 160 mg/kg b.w., with saline solution as a negative control and cyclophosphamide, a known mutagen and clastogen as a positive control at twenty four hour intervals during a seven day period.

Micronucleus test results showed a consistent dose-dependent pattern. Thus, with increase of the dose (10 mg/kg b.w., 90 mg/kg b.w. and 160 mg/kg b.w.) there was an increase in the frequency of micronuclei in polychromatic erythrocytes (4.88 ± 0.35; 8.32 ± 0.57; 11.75 ± 0.37) compared to the negative control (2.04 ±0.28). Using chromosome aberrations as an indicator of genotoxic potential, 8-Cl-cAMP in all three doses (10 mg/kg b.w.; 90 mg/kg b.w. and 160 mg/kg b.w.) produced karyotype transformation of mouse bone marrow cells. 8-Cl-cAMP induced structural chromosome aberrations as lesions (2.87 0.14; 4,37 0.14 i 5.25 0.35), interruptions (9±0.1; 12.37±0.26 i 13.37±0,33), ring chromosomes (3.62 0.21; 2.5±0,07 i 2.5±0.07), accentrics (10±0.49; 16.5±0.45 i 18.37±0.54) and

Robertsonian translocations (7.12±0.26 ;9 0.1 i 11.24 ±0.18) as well as numerical chromosome aberrations of the aneuploidal type (36.5± 0.74; 60.25±0.24 i 85.62±0.5) and polyploidy (7±0.24; 5.5±0.21 i 5.87±0.14). These results demonstrate the genotoxic potential of the investigated substance.

Key words: Antitumor agent 8-Cl-cAMP; genotoxicity; apoptosis; micronucleus test; cytogenetic in vivo test; numerical and structural aberrations

INTRODUCTION

Preclinical investigations of 8-Cl-cAMP, an analogue of cAMP, with strong antineoplastic activity *in vitro* and *in vivo* have given us new hope for treatment of various tumors. This is the first analogue of cAMP to be introduced to phase I clinical trials on patients with colon and breast tumors (Tortora et al., 1995).

Cyclic-adenosinemonophosphate (cAMP) is implicated in the regulation of growth in normal and malignant cells (Cho-Chung et al., 1989; Cho-Chung et al., 1990). This second messenger is also implicated in apoptotic cell death in lymphoid (Mc Donell et al., 1993) and myeloid cells (Duprez et al., 1993). The primary mediator of cAMP action in eukaryotic cells is cAMP-dependent protein kinase. There are two classes of cAMP-dependent protein kinase, designated as type I and type II, which contain distinct R subunits, but share common a C subunit (Ciardiello et al., 1990). Differential expression of these two distinct protein kinase isozymes, have been linked to the regulation of cell growth and differentiation (Cho-Chung et al., 1989). It has been shown that type I cAMP-dependent protein kinase and/or its regulatory subunit are overexpressed in cancer cell lines and primery tumors (Ciardiello et al., 1993). On the other hand increased R II or type II cAMP dependent protein kinase levels are present in normal differentiated cells and precede the cell growth arrest by cAMP analogues. The R subunits, R I and R II, have different sites on which cAMP analogues will bind. Depending on the substituents on the adenine ring, cAMP analogues selectively bind to site 1 (C-2; C-8 analogues) or site 2 (C-6 analogues). Unlike intracellular cAMP, the new class of site-selective cAMP analogues are able to selectively discriminate between the two cAMP binding sites present on R I and R II and to modulate the intracellular levels of regulatory subunits at micromolar concentrations. 8-Cl-cAMP, the most potent site-selective analogue (Cho-Chung et al., 1990) is able to down regulate the R I subunit by inducing degradation of the protein while up-regulating R II subunit expression at the transcription level (Rohlff et al., 1993). This potent modulating activity of 8-Cl-cAMP has shown an inhibitory effect on the growth of a wide variety of tumors *in vivo* and *in vitro* (Cho-Chung et al ., 1990).

On the basis of preclinical and toxicological studies of 8-Cl-cAMP on animal models (Dixit et al., 1993) phase I clinical trials have been conducted in order to define toxicity, maximal tolerated dose, drug plasma concentrations and immunological effects. All analyses were performed on patients that were refractory to standard treatments (Tortora et al., 1995). The literature at present available in

connection with cAMP and its analogues, has not provided us with one scientific paper or study concerning their genotoxic or mutagenic potential *in vivo* or *in vitro.* The pioneer step in genotoxicological research of cAMP analogues, as highly potent antitumor agents, was made by Bajić (1998) and Bajić et al. (1998; 1999).

Since 8-Cl-cAMP has been investigated as a new potential anti cancer drug with no previous studies of its mutagenic and clastogenc effects, we have conducted a study to estimate the genotoxicity of 8-Cl-cAMP using different doses in the micronucleus and cytogenetic *in vivo* assay.

MATERIAL AND METHODS

The genotoxic potential of 8-Cl-cAMP was estimated from micronucleus and chromosome aberration tests on bone marrow cells of BALB/c strain mice. The method by Schmid (1975) was used for the analysis of micronuclei (MN) in polychromatic erythrocytes (PCE) of the mouse bone marrow Cyitogenetic analysis was performed by the direct method of rinsing the marrow of long bones (femur and tibia) according to Hsu and Patton (1969), as modified by Zimonjić (1990). The investigated substance was tested at three experimental concentrations. The lowest concentration should correspond to its level in the environment, but, as this is a synthetic substance, the level of 10 mg/kg b.w. was taken on the basis of postnatal growth toxicity induced by 8-Cl-cAMP (Milutinović et al., 1996). The median concentration was established from the maximal tolerance dose, a dose which shows clinical manifestations of mild toxicity (loss of weight, diarea, ataxia, somnolence) (Aardema, 1994). The high concentration is a sublethal dose of 8-Cl-cAMP, 160 mg/kg b.w. based on preliminary toxicological investigation (acute toxicity) on BALB/c mice. Experimental designe for both *in vivo* tests included three groups: positive control, negative control and experimental groups. The experimental groups were divided into three sub-groups based on the chosen doses of 8-Cl-cAMP.

The negative control group was treated with physiological saline solution. A known mutagen, cyclophosphamide, in a dose of 40 mg/kg b.w. was used for the positive control group. All groups had equivalent numbers of animals per test. Thus, for the cytogenetic test 6 animals were used per dose/group (both sexes) and for the micronucleus test 8 animals were used per dose/group (both sexes). The animals were kept under uniform conditions (Bajić et al., 1994), housed under a 12/12h photoperiod at constant temperature (21^{o}C) with free access to standard laboratory chow and water. Cyclophosphamide and 8-Cl-cAMP were dissolved in physiological saline solution immediately before i.p. administration, and the volume injected was 0.01ml/g body weight. All animals received daily i.p. treatments for a seven day duration.

The results were statistically analysed by suitable computer programmes (Excel, Microstat Statistica) with the use of Student's t-test (Petz, 1981).

RESULTS AND DISCUSSION

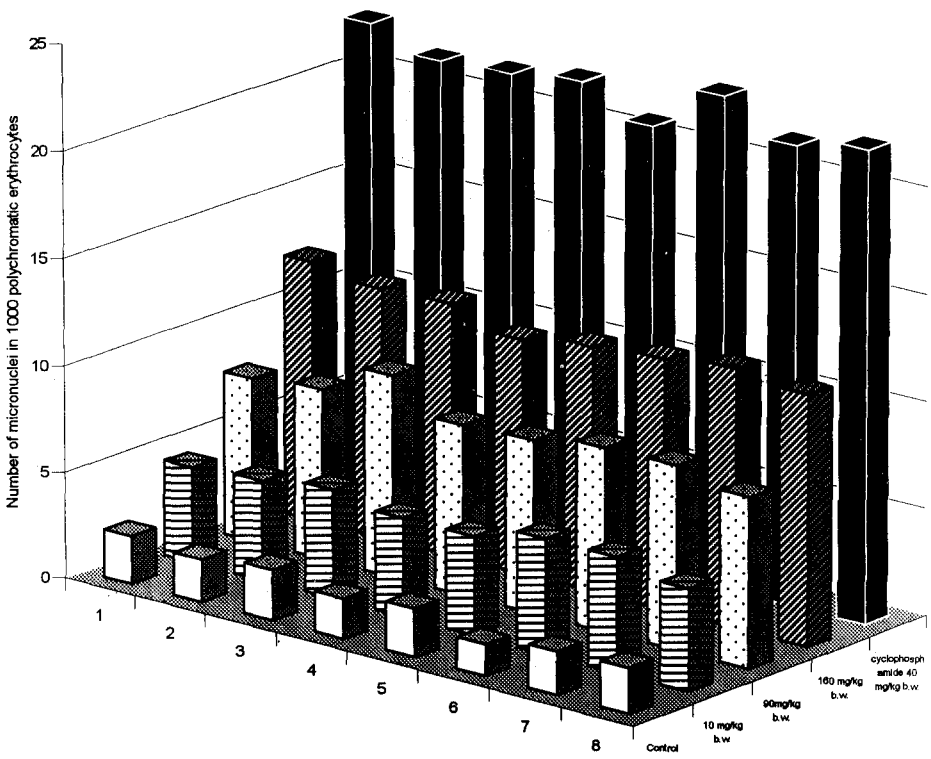
The administration of 8-Cl-cAMP showed an ability to induce micronuclei (MN) in polychromatic erythrocytes (PCE) of bone marrow of BALB/c mice (Table 1; Figure 1). Statistical analysis demonstrated a statistically highly significant difference ($p<0,001$) in micronuclei induction (4.88 ± 0.35; 8.32 ± 0.57; 11.75 ± 0.37) over the negative control (2.04 ± 0.28) at all three concentrations of 8-Cl-cAMP (10 mg/kg b.w.; 90 mg/kg b.w. and 160 mg/kg b.w.). Also, these results demonstrated a dose-response relationship between exposure to 8-Cl-cAMP and the frequency of MN in PCE. Even though tis dose-response relationship indicates 8-Cl-cAMP genotoxicity, we must not exclude the possibility of genotoxicity decrease (or elimination) by reducing the dose and retaining the anti-neoplastic effect of our investigated substance (Bajić, 1998; Bajić et al., 1998; 1999).

Table 1. Frequency of polychromatic erythrocytes (PCE) with micronuclei in bone marrow cells of control and experimental groups of strain BALB/c mice treated with increasing doses of 8-Cl-cAMP

Cycles repeated	Control	10 mg/kg b.w.	90 mg/kg b.w.	160 mg/kg b.w.	cyclophosphamide 40 mg/kg b.w.
1	2.25	4.5	7.7	12.2	22.5
2	2	4.7	8	11.75	21.5
3	2.37	5.12	9.5	11.87	21.7
4	1.87	4.62	8	11	22.1
5	2.25	4.62	8.12	11.5	20.8
6	1.47	5.37	8.62	11.75	23
7	2	5.37	8.62	12.12	21.5
8	2.12	4.75	8	11.75	22.1
X ± SD	2.04±0.28	4.88±0.35	8.32±0.57	11.74±0.37	21.86±0.71

Cyclophosphamide, an antitumor agent, used in treatment for a variety of tumors, is also employed as a positive control in a number of genotoxicological tests because of its known clastogenic and mutagenic features (Anderson et al., 1995). Comparing the frequency of MN in PCE at the 160 mg/kg b.w. dose of 8-Cl-cAMP (11.75 ± 0.37) and of cyclophosphamide at its therapeutic dose of 40 mg/kg b.w. (22.1 ± 0.71), we noticed that cyclophosphamide is two times more potent as an inducor of micronuclei than 8-Cl-cAMP. The same result can be more clearly presented as a genotoxic effect of 8-Cl-cAMP in relationship to our positive control. Namely, if the frequency of MN (21.9 ± 0.71) in PCE induced by cyclophosphamide (40 mg/kg b.w.) is taken as 100% (Table 1), then for the experimental group receiving 160 mg/kg b.w. of 8-Cl-cAMP (11.74 ± 0.37) there was a 52.9% level of genotoxicity, at 90 mg/kg b.w. (8.32 ± 0.57) 37.6% and for 10 mg/kg b.w. (4.88 ± 0.35) 21.4%. These values demonstrate that 8-Cl-cAMP

Figure 1. Frequency of micronuclei in polychromatic erythrocytes in mouse bone marrow cells of control groups and experimental groups treated with increasing doses of 8-Cl-cAMP



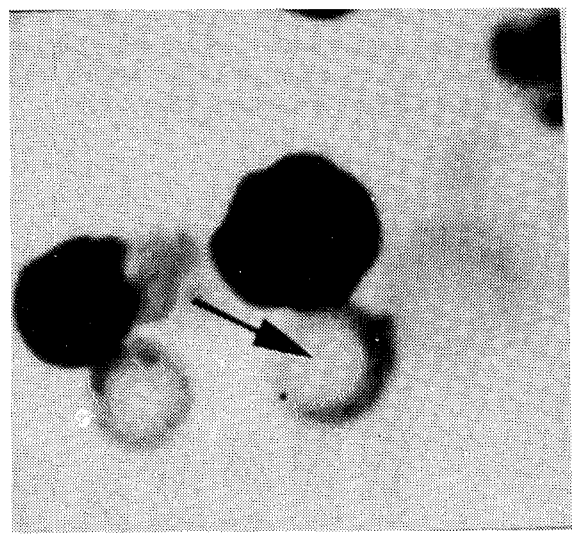


Figure 2. Micronuclei in polychromatic erythrocytes of BALB/c mice induced by 8-Cl-cAMP in the dose of 90 mg/kg b.w.

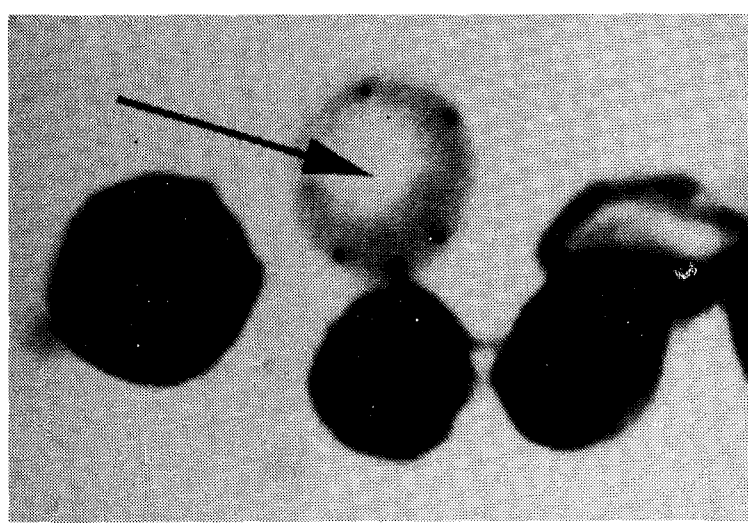


Figure 3. Micronuclei in polychromatic erythrocytes of BALB/c mice induced by 8-Cl-cAMP in the dose of 160 mg/kg b.w.

at 10 mg/kg b.w. is five times less potent in inducing MN in PCE than cyclophosphamide in its therapeutic dose range (40 mg/kg b.w.). The possibility of using

Figure 4. Percentage values of cytogenetic parameters in mouse bone marrow cells of control groups and experimental groups treated with increasing doses of 8-Cl-cAMP

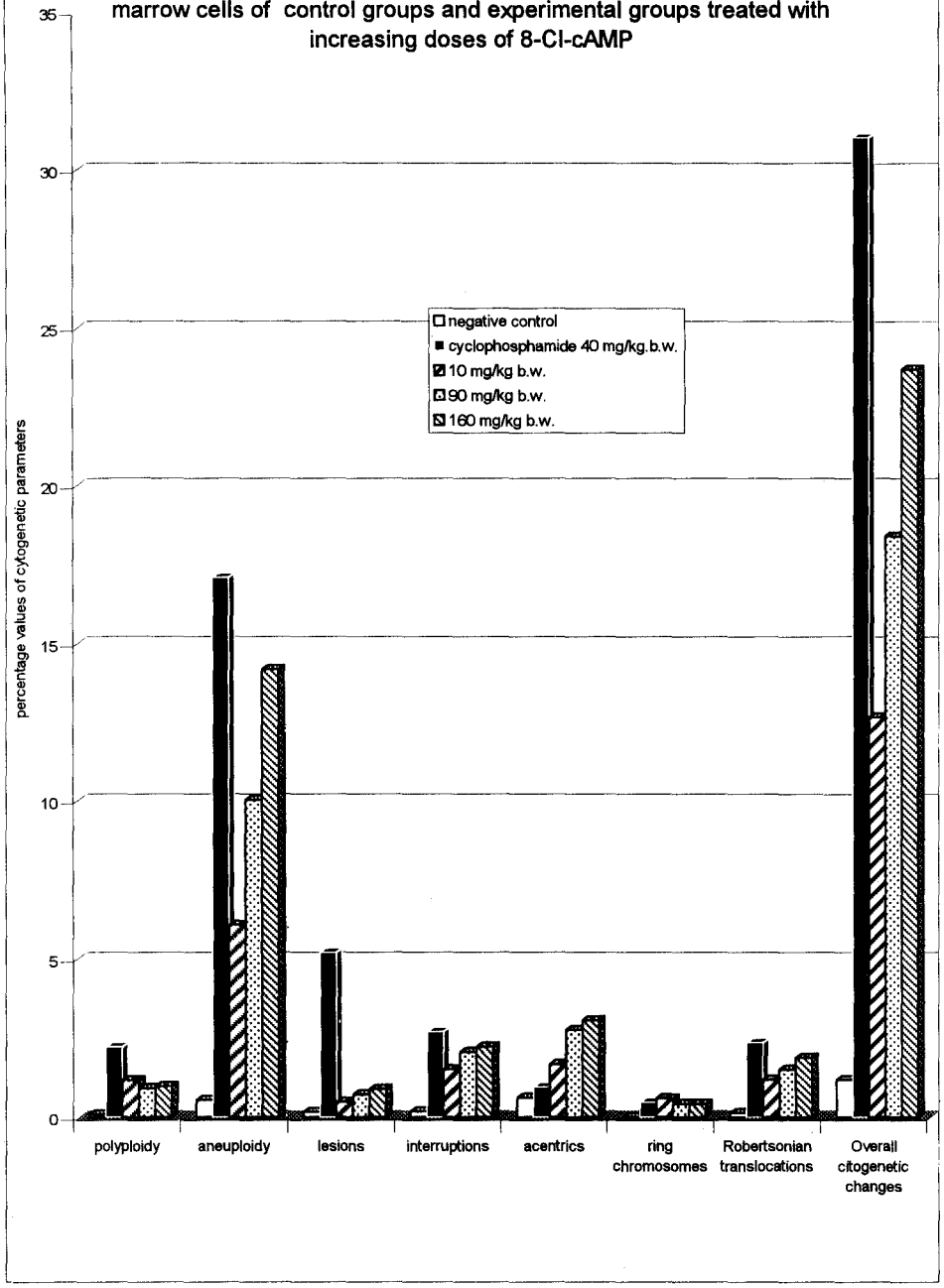


Table 2. Cytogenetic parameters of mouse bone marrow cells of control groups and experimental groups treated with increasing doses of 8-Cl-cAMP

Cytogenetic parameters	Negative control		Positive control cyclophosphamide 40mg/kg b.w.		10 mg/kg b.w. 8-Cl-cAMP		90 mg/kg b.w. 8-Cl-cAMP		160 mg/kg b.w. 8-Cl-cAMP90	
	X ± SD	%	X ± SD	%	X ± SD	%	X ± SD	%	X ± SD	%
Polyploidy	0.50±0.05	0.08	013.30±0.37	02.22	00.70±0.24	01.16	005.50±0.21	00.91	005.87±0.14	00.97
Aneuploidy	3.25±0.15	0.54	102.56±0.78	17.10	36.50±0.74	06.08	060.25±0.24	10.04	085.62±0.50	14.20
Lesions	1.00±0.08	0.16	031.39±0.52	05.22	02.87±0.14	00.47	004.37±0.14	00.72	005.25±0.35	00.87
Interruptions	1.12±0.07	0.18	016.20±0.38	02.70	00.90±0.01	01.50	012.37±0.26	02.06	013.37±0.33	02.22
Accentrics	0.37±0.07	0.61	005.70±0.03	00.95	10.00±0.49	01.66	016.50±0.45	02.75	018.37±0.54	03.06
Ring hromosomes	0.00±0.00	0.00	002.80±0.02	00.47	03.62±0.21	00.60	002.50±0.07	00.41	002.50±0.07	00.41
Robertsonian translocations	0.70±0.01	0.13	014.17±0.41	02.35	07.12±0.26	01.18	000.90±0.10	01.50	011.24±0.18	01.87
Overall Cytogenetic shanges	6.99±0.55	1.17	186.15±1.7	31.03	76.10±2.17	12.68	110.49±1.47	18.40	142.23±2.10	23.70

lower doses to decrease or even eliminate the genotoxic effect of 8-Cl-cAMP is important for assessment of the therapeutic risk. Almost all anticancer drugs used today have the potential to induce secondary tumors after initial treatment (Pedersen et al., 1988; Pedersen-Bjergaard and Philip., 1989), so the possibility of reducing the genotoxicity of 8-Cl-cAMP while it decreases the expression of c-myc and ras oncogenes in various cancer cells *in vitro and in vivo* (Cho-Chung, 1990) may give a therapeutic benefit of 8-Cl-cAMP over many known anticancer drugs. However when extrapolating any risk assessment from mice to humans we have to stress the importance of interspecies variability, that is, the difference between species of enzymatic systems which are responsible for metabolic activation, distribution and elimination of the investigated substance (Tortora et al., 1995; Rusov et al., 1996).

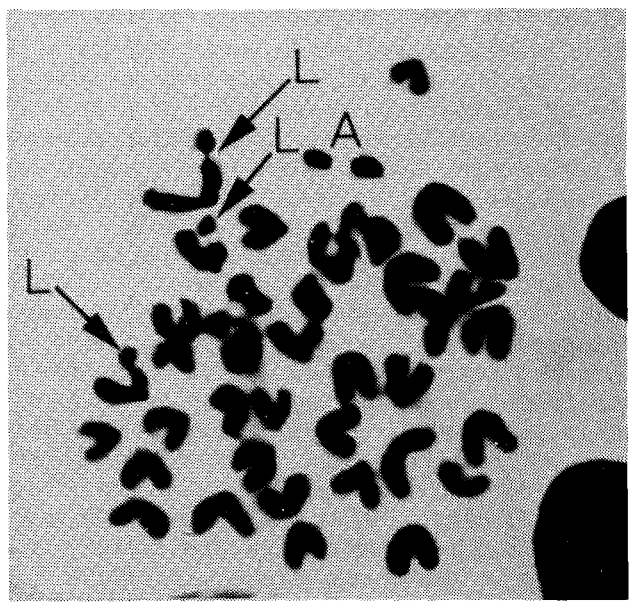


Figure 5. Aberrant karyotype with lesions and accentrics in bone marrow cells of BALB/c mice treated with 8-Cl-cAMP in the dose of 160 mg/kg b.w.

It was established by cytogenetic analysis that during a seven day (i.p.) treatment with increasing doses of 8-Cl-cAMP (10 mg/kg b.w.; 90 mg/kg b.w. and 160 mg/kg b.w.). there was an increase of frequency of numerical and structural chromosome aberrations in mouse bone marrow cells over negative control.

Numerical aberrations of the aneuploidal and polyploidal type are the consequence of abnormal segregation of chromosomes during cell division. Abnormal segregation can arise by action of the investigated substance on kinetochor proteins which affect the centromeric region or mitotic spindle, so the

chromosomes lag due to spindle dysfunction and consequently a change in chromosome number occurs during the cell cycle which is exhibited as numerical chromosome aberrations (Legator et al.,1994; Soldatović et al., *1994;* Stanimirović.,1995; Marković, 1996; Stanimirović et al., 1997; Bajić, 1998; Stanimirović et al., 1998; Marković,1999). A relatively high frequency of aneuploidy and polyploidy was noticed in the bone marrow cells of animals treated with in all three doses of 8-Cl-cAMP. Thus the dose of 10 mg/kg b.w. induced aneuploidy at a level of 6.08%, polyploidy at 0.93%; 90 mg/kg b.w. led to 10.04% aneuploidy and 0.93% polyploidy while 160 mg/kg b.w. gave 14.2% aneuploidy and 0.97%. polyploidy compared with 17.9% aneuploidy and 2.22% polyploidy for the positive control (Table 2; Fig. 4). As only the dose of 10 mg/kg b.w. of 8-Cl-cAMP can be extrapolated to the therapeutic dose range used for humans (Tortora et al., 1995), we can note that 8-Cl-cAMP is almost three times less potent in induction of aneuploidy and polyploidy than cyclophosphamide in its therapeutic dose range.

8-Cl-cAMP showed the ability of inducing the following structural chromosome aberrations: lesions, interruptions, ring chromosomes, accentrics and Robertsonian translocations. Structural aberrations of the lesion and interruption type can be a sensitive and accurate parameter in estimating genotoxicity (Broger et al., 1982). The progressive increase in both types of lesion in the experimental groups and levels found in the positive control group (Table 2.)indicate their relative potency in inducing these lesions (Bajić, 1998). Also, all investigated doses of 8-Cl-cAMP induced chromosome changes of the accentric, ring chromosome and Robertson translocation type in mouse bone marrow cells (Table 2: Fig 4). Thus, highly significant differences were found in the overall percentage of cytogenetic changes in cells of the negative control group (1.17%) and experimental groups treated with three different doses of 8-Cl-cAMP 12,68%, 18.41% and 23.7% respectively. Highly significant differences ($p<0.001$) were also observed between all experimental groups and cyclophosphamide. Even though a high dose regime is required for evaluating potential genotoxic effects of any substance (Aardema,1994), our doses (90 mg/kg b.w. and 160 mg/kg b.w.) used in vivo can result in pathologies that would be eliminated in lower dose ranges. Therefore it can be said that high dose regimes acted saturably on the elimination, metabolism and repetitive mechanisms (Crouch et al., 1979).

For years it has been known that cytotoxic effects of chemotherapeutic drugs are explained as the conseqence chromosome and chromatid damage (Soldatović et al., 1994; Stanimirović.,1995; Marković, 1996; Stanimirović et al., 1997; Bajić, 1998; Stanimirović et al., 1998; Marković,1999), but in recent years it has emerged that cytotoxic effects of chemotherapeutic agents can result from the programmed cell death apoptosos (Corcoran, et al., 1994; Boe et al., 1995). As 8-Cl-cAMP and its active metabolite 8-Cl adenosine are strong inducors of apoptosis, the relationship between genotoxic effects of cytotoxic drugs and their apoptotic potential, that is the portion of 8-Cl-cAMP genotoxicity engaged in the induction of apoptosis should be evaluated. The early phase of apoptosis starts with chromosome fragmentation. Three types of chromosome fragmentation can be distinguished: intranucleosomal cleavage of DNA; degradation of DNA to long fragments 50-300 kb in length and single-strand breaks (SSB) of DNA (Marini et

al., 1996). The role of SSB in the process of apoptosis is still an open question, that is, can SSB be an early signal for apoptotic induction.

The results of our investigation have shown that in the overall picture of registered changes on chromosomes, the highest percentage of aberrations are of the lesion, interruption and accentric type (figure 5). If we correlate these results with the inductive capability of 8-Cl-cAMP to increase the frequency of MN in PCE and the 8-Cl-cAMP apoptotic potential, then we can pose a question : Is the genotoxic effect of 8-Cl-cAMP a primary signal for apoptotic induction or is the induction of apoptosis an independent process? Our results strongly suggest that there is a correlation of 8-Cl-cAMP genotoxicity and its apoptotic potential, which will have great importance for the development and use of 8-Cl-cAMP as an anticancer agent. It is also important to stress that correlative relationships between genotoxicity and apoptosis will open new strategies for the use of various cytotoxic drugs by modulating the process of PCD in tumors that are refractory to previous treatments (Bajić, 1998).

The experimental results demonstrate a correlation between 8-Cl-cAMP cytotoxicity and genotoxicity. One of the possible reasons is that the plateau of overall cytogenetic changes already exists at the MTD dose level (90mg/kg b.w.) where animals clinically express signs of mild toxicity. However, is the action of 8-Cl-cAMP direct or indirect, that is, are changes on chromosomes and the induction of MN a consequence of its active metabolite 8-Cl-adenosine, or both, is a question to be answered (Cornelis et al., 1992). The results from other preclinical investigations of 8-Cl-cAMP demonstrate a complicated action of 8-Cl-cAMP in various tumor cell lines in vitro and in vivo (Cummings et al., 1996). Our results demonstrate indisputable evidence of the genotoxic potential of 8-Cl-cAMP on bone marrow cells of BALB/c mice strain. However, correlation between dose-response and the existence of inter and intra-species genetic variability with regard to the metabolism of 8-Cl-cAMP (Tortora et al., 1995; Bajić, 1998) does not let us extrapolate any conclusion about assessment the carcinogenic and mutagenic risk of 8-Cl-cAMP in humans 8-Cl-cAMP is the first analogue to be evaluated in phase I clinical trials on patients with colon and breast tumors. The results of our investigation, suggest novel molecular-biological in vitro genotoxicological studies, especially in relation to apoptosis in order to evaluate the overall genotoxic risk of 8-Cl-cAMP treatment.

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ISPITIVANJE GENOTOKSIČNOSTI 8-Cl-cAMP U DVA IN VIVO TESTA NA MIŠEVIMA SOJA BALB/c

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SADRŽAJ

Antitumorski preparat 8-Cl-cAMP (8-hloro-ciklični adenozin monofosfat) je najpotentniji analog AMP koji deluje primarno na modulaciju cAMP - zavisne protein kinaze dovodeći do inhibicije regulatornih subjedinica. Rezultat takvog dejstva je smanjenje dominantnog tipa PK-I protein kinaze u kanceroznim ćelijama nad drugim tipom PK-II protein kinaze, u odnosu na međusobni nivo PK-I I PK-II koji se nalaze u normalnim ćelijama. Ciklični 8-Cl-AMP je analog koji ima izuzetnu anti-neoplastičnu aktivnost sa efektom restauracije, diferencijacije i reverzne transformacije kanceroznih ćelija miša i čoveka. Sa stanovišta mogućeg mutagenog i genotoksičnog efekta 8-Cl-cAMP nije dovoljno istra'en. Zato je i cilj ovog rada bio da se primenom citogenetičkog i mikronukleus testa, na ćelijama kostne sr'i miša BALB/c soja, ispita genotoksični efekat 8-Cl-cAMP u tri dozna re'ima (10 mg/kg t.m; 90 mg/kg t.m. i 160 mg/kg t.m.). Pored eksperimentalnih grupa

životinja u eksperimentu su uspostavljene i negativna kontrola koju su činile jedinke tretirane sa fiziološkim rastvorom, kao i pozitivna kontrola životinja koje su tretirane sa poznatim klastogenom i mutagenom-ciklofosfamidom u dozi od 40 mg/kg t.m. Rezultati ispitivanja pokazuju konzistetni dozno-zavisni obrazac primenom mikronukleus testa. Sa rastom doze (10 mg/kg t.m ; 90 mg/kg t.m. i 160 mg/kg t.m.) raste i broj mikronukleusa u polihromatofilnim eritrocitima (4,88 ±0,35; 8,32±0,57; 11,75±0,37) u odnosu na kontrolnu grupu (2,04±0,28). Testirane rastuće doze 8-Cl-cAMP u citogenetičkom testu *in vivo* pokazuju sposobnost transformacije kariotipa ćelija kostne sr'i BALB/c miševa u vidu strukturnih hromozomskih aberacija tipa lezija (2,87±0,14; 4,37±0,14; 5,25±0,35), prekida (90,1; 12,37±0,26; 13,37±0,33), ring hromozoma (3,62±0,21; 2,5±0,07; 2,5±0,07), acentrika (100,49; 16,5±0,45; 18,37±0,54) i Robersonovih translokacija (7,12±0,26; 90,1; 11,24±0,18) i numeričkih hromozomskih aberacija tipa aneuploidija (36,5±0,74; 60,25±0,24; 85,62±0,5) i poliploidija (70,24; 5,5±0,21; 5,87±0,14) što ukazuje na postojanje genotoksičnog potencijala ispitivane supstance.